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C.ELEGANS HOST CELL FACTOR (CeHCF) ASSOCIATES WITH SIR-2.1 TO MEDIATE TARGET SPECIFICITY OF DAF-16 AND LIFESPAN

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ABSTRACT

The programmed aging process, controlled by a complex gene network, exists in all living organisms. In past decades, studies on the model organism, the nematode worm *Caenorhabditis elegans* (*C. elegans*), have revealed some highly conserved molecular pathways involved in lifespan regulation. After entering nucleus, DAF-16 cooperates with corresponding cofactors to achieve its specificity and triggers the transcription of certain subsets of its targets. Here we report that a novel DAF-16 co-factor CeHCF (C.elegans Host Cell Factor) mediate lifespan by regulating NAD+-dependent protein deacetylase, SIR-2.1.

Lifespan assay using *Cehcf* RNAi revealed that the lifespan extension caused by loss of *Cehcf* could be fully suppressed by loss of *sir-2.1*. *Cehcf* RNAi also fails to further extend the lifespan of long-lived SIR-2.1 overexpression mutants in the lifespan assay, which indicates the epistatic relationship between *Cehcf* and *sir-2.1*. Furthermore, transgenic animals generated by microinjection with additional copies of *Cehcf* and *sir-2.1* showed a significant shortened lifespan comparing to *sir-2.1* overexpression mutants. Thus overexpression of CeHCF could fully suppress the lifespan elongation in the SIR-2.1 overexpression mutants, which suggests CeHCF may negatively regulate SIR-2.1. Moreover, realtime pcr revealed that the transcriptional level of *sod-3*, a target of DAF-16, is not directly regulated by CeHCF/SIR-2.1, which suggests that CeHCF/SIR-2.1 may function to limit the specificity of DAF-16 rather than simply to regulate transcriptional activity of DAF-16. Overall, this study revealed a novel genetic pathway, CeHCF/SIR-2.1/DAF-16, and further research on this pathway would largely facilitate our understanding of the aging process.

DEDICATION

I would like to dedicate this work to my parents, Shuqiao Yao and You Chen, for their continuous support, encouragement and love.

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LIST OF ABBREVIATIONS

AMPK…… AMP-activated Protein Kinase

CeHCF…… *C.elegans* Host Cell Factor

CR…….. Calorie Restriction

DAF-16…….... Abnormal Dauer Formation Protein 16

ERCs……. Extrachromosomal DNA Circles

FOXO…….. Forkhead Box O

HDAC…… Histone Deacetylase

HMT…….. Histone Methyltransferase

HSV…….. Herpes Simplex Virus

IIS…… Insulin/Insulin-like Signal

JNK……. Jun N-terminal Kinase

MST1…….. Macrophage Stimulating 1

MAPK……. Mitogen-activated Protein Kinase

NLS…… Nuclear Location Signal

PBK…… Protein Kinase B

qRT-PCR……. Quantitative Realtime Poly Polymerase Chain Reaction

CHAPTER ONE

INTRODUCTION

The desire to live longer and remain healthy during later years is always present in human society. Even though the life expectancy is increasing every year, chronic disease, such as cardiovascular disease, cancer, lung disease, and diabetes induced by the act of aging is still the major cause of death all around the world [1, 2]. Thus, understanding of the mechanism of aging will bring insights to those disease treatments and in turn greatly benefit the improvement of human health.

To better investigate molecular mechanism of aging, small nematode *C. elegans* is commonly used as the model genetic organisms, because it is a simple multicellular eukaryotic which shares a great genome similarity with human. By studying *C. elegans*, many highly conserved signaling pathways have been revealed, among which the DAF-16/insulin-like signaling pathway is of best understanding [3-5]. DAF-16/insulin-like signaling pathway is highly conserved in various species [3-7]: When DAF-2 insulin receptor sensed insulin-like peptides [8], the DAF-16/FOXO transcription factor would be phosphorylated by PI3/AKT/SGK kinase cascade and retained in the cytoplasm resulting in a regular lifespans [9-11]. Certain environmental stresses or reduced insulin-like signaling could trigger nuclear translocation of DAF-16, which results in increased transcription of genes that are responsible for longevity and stress resistance [12-22]. For example, heat shock stress, oxidative stress, and starvation could induce corresponding protein kinases, such as JNK, MST1, and AMPK, to mediate the lifespan and stress resistance by modifying DAF-16 [12-20]. RLE-1 also controls the ubiquitination level of DAF-16, which in turn

affects lifespan in worms [23].

Moreover, many transcriptional cofactors work with DAF-16 in response to different upstream stimulus [17-22]. Notably, among these transcriptional cofactors, SIR-2.1, a conserved NAD+-dependent protein deacetylase, may play a critical role in regulating DAF-16-mediated aging process [24-28]. SIRT1, the mammalian homolog of SIR-2.1, modulates transcriptional activities by deacetylating FOXOs [29, 30]. Extra copies of *C. elegans* SIR-2.1 enhance longevity and stress resistance in a manner that is dependent on DAF-16/FOXO and 14-3-3 scaffold proteins [27, 28].

This study attempted to address the molecular basis for the longevity function of *C. elegans* host cell factor (CeHCF). Previous studies show that loss of CeHCF could extend lifespan and enhance stress resistance in a DAF-16 dependent manner [31]. Here we report that by genetic approaches, we identified that SIR-2.1 is also involved in CeHCF-mediated lifespan regulation. Deciphering the cooperation code among CeHCF, SIR-2.1 and DAF-16 will greatly facilitate our understanding of aging regulation.

CHAPTER TWO

LITERATURE REVIEW

DAF-16: the center of the aging regulating network

DAF-16 belongs to the forkhead transcriptional factor family. It shares share a high similarity in the DNA binding domain with its mammalian homologs FOXO1, FOXO3a, FOXO4 [32, 33] [Figure 2-1]. There are total seven isoforms of DAF-16 in worms and function of each is not clear [33]. DAF-16 was initially indentified as a factor that caused dauer defective phenotype upon its mutation [32], but the two major isoforms DAF-16A1 and DAF-16B seem to have distinct roles in aging regulation [34].

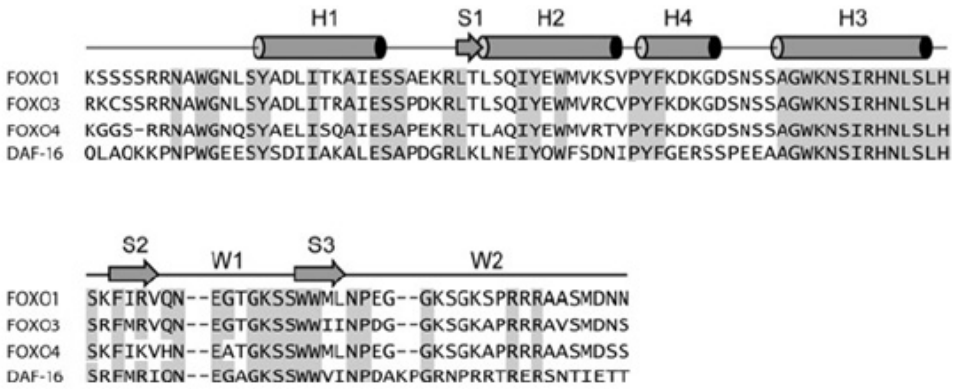


Figure 2-1. DAF-16 shares a highly conserved DNA binding domain with FOXO1, FOXO3, and FOXO4. Conserved DNA binding domain “winged helix/forkhead domain” is characterized by three α-helices (H1, H2, H3), three small β-strands (S1, S2, S3), and two wing-like loops (W1 and W2). [revised from 33].

DAF-16 was reported to control longevity in *C. elegans* mainly through the insulin-like metabolic pathway [4, 5]. It is highly conserved in various species [3-7]. When DAF-2 insulin receptor sensed insulin-like peptides [8], the DAF-16/FOXO would be phosphorylated by PI3/AKT/SGK kinase cascade and the phosphorylated DAF-16/FOXO will be retained in the cytoplasm so the longeivity and stress resistance gene regulated by DAF-16/FOXO could not be expressed [9-11]. While reduced insulin-like signaling could cause dephosphorylation of DAF-16/FOXO in the AKT site, which results in increased lifespan and stress resistance [9-11]. However, the role of DAF-16 and insulin-like signaling pathway may be varied in different tissues [35]. DAF-28 which encodes an insulin-like peptide, is expressed in many sensory neurons [8], but the neuronal activity of *daf-16* is more important for dauer formation rather than longevity regulation comparing to its intestinal activity [35]. It is still unclear that how crosstalk between different cells contributes to the lifespan regulation but the possible mechanism might involve a transmittable hormone.

Besides insulin-like signaling, certain environment stresses could induce corresponding factors to mediate the lifespan and stress resistance by modifying DAF-16. For example, germline signal could affect lifespan depending on the function of DAF-16 [36], but it is independent of IIS pathway since loss of germline could further extend lifespan in *daf-2* mutants [37]. Lipophilic hormone signaling by *daf-9* and *daf-12* are reported to play key roles in the communication between the reproductive system where the germline signal produced and the intestine where the DAF-16 is mainly located in [21, 38]. Diminution of DAF-12 ligands could abolish germline longevity, and reduce FOXO nuclear accumulation [21, 22]. Except for the germline signal, in

response to heat shock signal, JUN kinase (JNK) could also affect lifespan and stress resistance through DAF-16. Overexpression of JNK could trigger phosphorylation and nuclear translocation of DAF-16, which in turn results in the lifespan extension and increased stress resistance [12-14]. Similar to JNK, MST1 could phosphorylate FOXO in mammalian cells, resulting in dissociation between FOXO and 14-3-3 proteins, which facilitates nuclear translocation of FOXO where it initiates cell apoptosis [15]. Overexpression of the *C. elegans* homolog MST extends lifespan depending on DAF-16 as well [15]. Another DAF-16 related kinase is AMP-activated protein kinase (AMPK), which is a fuel sensor, sensitive to AMP/ATP ratios. Loss of function of AMPK suppresses *daf-2* longevity, while overexpression extends lifespan [16].

Nuclear translocation of DAF-16/FOXO is considered as the central event in lifespan regulation. In mammals, the FOXOs could be phosphorylated on multiple threonine and serine residues. One major phosphorylation site of FOXOs is PKB site which determines the cellular location of FOXOs [39, 40]. When the activity of PKB is at a relative low level, the rate of importing FOXOs is higher than the rate of exporting of FOXOs, so FOXOs primarily in the nucleus [39, 40]. While activated PKB could phosphorylate FOXOs and the phosphorylated FOXOs would have a higher binding affinity to the 14-3-3 proteins, resulting in FOXO protein detach from the DNA and being retained to the cytosol [39-41]. 14-3-3 proteins also bind to the nuclear location signaling site of FOXO in order to prevent FOXO from re-entering the nucleus [34, 41]. In *C.elegans*, AKT phosphorylation deficiency induces nuclear accumulation of DAF-16, but it was not sufficient to induce dauer formation or lifespan extension [34], which suggests there should be other elements that co-regulate binding activity of DAF-16. For instance, many aging signaling,

such as pathogen, UV, and oxidative-stress alter DAF-16 transcriptional output by inducing DAF-16 to associate with a conserved nuclear factor, SMK-1 [17]. Overexpression of HSF-1, a heat shock transcription factor In response to specific heat shock stress, which induces various heat shock chaperones, increases heat resistance and extends lifespan in a DAF-16-dependent manner [18, 19]. Additionally, BAR-1, a homolog of β-catenin in *C. elegans,* physically associates with DAF-16 in response to oxidative stress [20]. Besides phosphorylation, studies have shown that acetylation level of FOXO could be regulated by cAMP response element binding (CREB)-binding protein (CBP) and deacetylase SIRT1, and both are important regulators of FOXO-mediated transcriptional activity [29, 30]. SIR-2.1, the ortholog of SIRT1 in *C.elegans*, affects aging partially depending on DAF-16. Overexpression of *sir-2.1* could extend lifespan in a DAF-16-dependent manner [28]. However, direct evidence is still missing that *sir-2.1* modulates transcriptional activity or target specificity of DAF-16 through its deacetylase enzyme activity.

The downstream targets of DAF-16 include genes that participate in various cellular events. The first few genes that were identified as DAF-16 targets include superoxide dismutase (SOD-3) [42], metallothioneine (MTL-1) [43], transmembrane tyrosine kinase (OLD-1) [44], SCP-like extracellular protein (SCL-1) [45], small heat shock proteins [46] and raptor (DAF-15) [47]. Moreover, by employing cDNA microarrays or the chromatin immunoprecipitation (ChIP)-based cloning strategy, various other DAF-16 targets were identified. Those targets generally could be classified into antioxidant genes, metabolic genes, small heat shock protein genes, and antibacterial genes [48, 49]. The longevity function of these genes could be derived from the general idea that an enhancement in cellular defense results in extended lifespan.

The large number of direct target genes brought into a question about the specificity of DAF-16: how could DAF-16 recognize the specific targets in response to corresponding signals? Recent studies about the physical structure of FOXOs might be able to provide a few clues. As members of the FOX family, all FOXOs contain a conserved DNA-binding domain (forkhead domain) with a winged helix fold that recognizes DNA [50]. This domain is composed of three α-helices, three β-strands, one wing called “wing 1” and the C-terminal α-helix called “wing 2” [50]. Since both phosphorylation site of PKB (Ser253) and acetylation sites of CBP (Lys242 and Lys245) are located in C-terminal [51], it is reasonable to propose that the post-translational modifications on C-terminal wing 2 might help to determine variable DNA recognition specificity FOXOs by either introducing a negative charge which alters the DNA-binding characteristics, or disrupting protein-DNA contacts in this region [46-48]. Indeed, flexibility of wing2 in FOXO1 affects its DNA recognition specificity [52]. Ser212, Ser218, Ser234 and Ser235 in FOXO1 are reported to interact with the phosphate backbone of the DNA and phosphorylation on these four sites by MST1 blocks DNA binding [53]. However, it shows MST1 phosphorylation on FoxO1 and FoxO3 promotes nuclear accumulation of FOXOs and increases expression of FOXOs downstream genes [15]. Therefore, it might require additional steps and other co-factors before FOXOs could recognize the MST1 mediated targets. On the other hand, DNA binding activity of FoxO1 could not be affected by Akt and CDK1/2 phosphorylation [53], which is consistent with the previous observations that phosphorylation by Akt regulates FoxO1 majorly by disrupting interaction between DAF-16 and 14-3-3 proteins. Acetylation of Lys245 and Lys248 in wing 2 by CBP/p300 reduces DNA binding affinity of FOXO1, which could, in turn, alter transcriptional activity of the

protein [53]. All of these observations support the hypothesis that post-translational modification in the wing 2 regions could affect DNA binding affinity of FOXOs.

SIR-2.1 plays multiply roles in aging control

SIR-2.1, the closest homolog of yeast SIR2 in worms, is a NAD+-dependent protein deacetylase. There are four SIR2 homologs in the nematode, SIR2.1-SIR2.4, and seven in mammals, SIRT1-SIRT7. The function of SIR-2.1 in lifespan regulation is quite complex, in both DAF-16-depedent or -independent manner.

It is well established that the calorie restriction diet could extend lifespan and prevent many aging associated late-onset diseases. In 1935, researchers found that the lifespan of laboratory rodents could be increased up to 50% by reducing their food intake from 30–40% [54]. Recent studies have uncovered that SIR2 and SIR2-like proteins (also called sirtuins) play an essential role in CR response. In the budding yeast *Saccharomyces cerevisiae*, reducing the available sugar in the medium could extend the replicative lifespan (the number of daughter cells that one mother cell can produce) in a SIR2 dependent manner [55]. The molecular mechanism behind this phenomenon could be that SIR2 inhibits the generation of extrachromosomal ribosomal DNA circles (ERCs) and protects cells from oxidative damages [25, 55, 56]. However, SIR2 is not required for chronological lifespan (the length of time that cells survive in a nondividing state) extension by CR [57]. These two contradictory roles of SIR2 brought the idea that SIR2 might play different roles in mitotic cells and post-mitotic cells, especially in higher multi-cellular eukaryotes. It was reported that *sir2* deletion mutants show increased expression of many genes involved in DNA repair and stress resistance, which are also activated by severe CR

[57]. Furthermore, in SIR2 deficiency, like severe CR, results showed increased resistance to heat and oxidative damage and elevated genomic stability [57]. Thus, the role of SIR2 in regulating CR response in yeast is not constant in response to different stress conditions.

Meanwhile, the link between SIR2 and FOXO was proven in many organisms. Increased dosage of SIR2 in yeast boosts longevity depending on the activity of FOXO [24]. And this function was also conserved in worms and flies [26, 28]. Extra copies of *C. elegans* sir-2.1 enhance longevity and stress resistance, in a manner dependent on DAF-16 and 14-3-3 scaffold proteins [27, 28]. In response to stress, SIR-2.1, 14-3-3 protein and DAF-16 form a nuclear complex that activates DAF-16 transcriptional targets such as *sod-3*, which also suggests it may acts in parallel to insulin-like signaling pathway [27]. In mammals, SIRT1 was also reported to deacetylate FOXO4 and FOXO1 [29, 30]. However, the relationship between SIR-2.1 and DAF-16 is not completely epistatic. UNC-13, a regulator of neurotransmitter release, was reported upstream of DAF-2. The lifespan assay of the mutant with loss of both alleles of *unc-13* (s69 and e450) shows a significant extension of lifespan, but this longevity could only be partially suppressed by lack of either DAF-16 or SIR-2.1; while the lifespan of the *daf-16; unc-13; sir-2.1* triple mutants is similar to wild type [58]. The partial overlapping of SIR-2.1 and DAF-16 could also be supported by their tissue specificity. Transgenic worms with SIR-2.1::GFP shows that the SIR-2.1 localize with DAF-16 in the nervous system and hypodermis, but also express in many pharyngeal cells alone [35, 58]. Meanwhile, there is no SIR-2.1 in the intestine, where DAF-16 is primarily existing and critical for insulin/IGF-1 pathway [35].

SIR-2.1 may also act in DAF-16-independent manner. A common sirtuin activator

resveratrol [59], one of the salutary compounds found in red wine, could extend the lifespan of yeast, worms, flies, and perhaps mice, in a sirtuin-dependent manner [60, 61]. In *C.elegans*, resveratrol induces *abu-11* and other components involved in endoplasmic reticulum (ER) stress response [62] in a DAF-16 -independent manner [63].

CeHCF mediates longevity in DAF-16 dependent manner

The host cell factor HCF-1 has been well known for its ability to stabilize the the herpes simplex virus (HSV) transactivator protein VP16-induced complex [64]. It contains several conservative domains: a Kelch domain, basic and acidic regions that are enriched respectively in basic and acidic residues, two fibronectin type 3(Fn3) repeats and a C-terminal nuclear localization signal (NLS) [60, 61]. A 2035-amino-acid precursor of HCF-1 undergoes a proteolytic process to generate amino- (HCF-1N) and carboxy- (HCF-1C) terminal subunits, and the stable association between these two units forms a heterodimeric complex to make the mature human HCF-1 [65, 66]. Besides the VP-16-induced complex, HCF-1 also binds to two human protein complexes that are involved in histone modification, the Sin3 histone deacetylase (HDAC) complex and the trithorax-related Set1/Ash2 lysine 4 histone H3 methyltransferase (HMT) [67]. HCF-1 is also a key regulator of cell proliferation, which can promote G1/S transaction by recruiting the HMT to activate E2F1 while employing the HDAC to repress E2F4 [68]. HCF-1N and HCF-1C have separate functions in G1- and M-phase progression: In the absence of HCF-1N-subunit function, mammalian cells enter a stable G1-phase arrest [60, 64, 65], while in the absence of the HCF-1C, cells exhibit multiple M-phase defects such as chromosome segregation, defective regulation of histone H4 lysine 20 (H4K20) methylation, and finally

resulting in multinucleated cells [69-72].

The homolog of HCF-1 in *C.elegans* is CeHCF, which conserves only the Kelch domain and Fn3 domain with NLS [73]. It does not retain the heterodimeric structure of proteolytically processed polypeptides [73]. CeHCF can stabilize the VP16-induced complex as its mammalian homolog [70]. The chromatin modification function of CeHCF is also conserved in both mammalian cells and *C.elegans:* as loss of CeHCF results in reduced histone H3S10 phosphorylation status [74]. However, the structure difference indicates that CeHCF may participate in controlling other life activities. A recent research shows one of its functions is in lifespan regulation [31]. It reports that loss of CeHCF caused lifespan extension of up to 40% and increased resistance of paraquat and cadmium exposure [31]. Meanwhile, this function of CeHCF fully depends on DAF-16 [31], because elongation of lifespan caused by *Cehcf* depletion can be fully suppressed by inactivation of *daf-16*[31]. In the absence of CeHCF, more DAF-16 is enriched on promoters of its target genes *sod-3* and *mtl-1*, and the expression level of a subset of DAF-16 regulated genes is enhanced [31]. Co-IP experiments also proved that CeHCF and DAF-16 have physical interaction in worms [31]. These results together indicate that Ce*hcf* is a novel upstream regulator of *daf-16*.

Considering the close relationship between SIR-2.1 and DAF-16, it is a logic assumption that CeHCF might also interact with SIR-2.1 to regulate longevity. Indeed, CeHCF is located in the nuclei of most somatic and germline cells in wild-type worms, which largely overlaps with SIR-2.1 [31]. Besides, the lifespan extension by loss of CeHCF could be fully suppressed by SIR-2.1 deletion (our lab data). The mechanism behind this might be derived from the physical

interaction between SIR-2.1 and CeHCF, which has been proved by *in vivo* co-immunoprecipitation (our lab data). However, the DAF-16/SIR-2.1/CeHCF super protein complex was not detected *in vivo* (our lab data). Previous studies show that the DAF-16/SIR-2.1 complex could only be detected under certain stress with 14-3-3 proteins [27]. Thus, it is possible that DAF-16/CeHCF and SIR-2.1/CeHCF protein complexes individually exist in the cells. The detailed machinery of CeHCF-modulated longevity still requires further investigation, results of which could largely help the understanding of aging mechanism involving DAF-16 and SIR-2.1.

CHAPTER THREE

MATERIALS AND METHODS

C.elegans strains

The strains used in this study were as follow: wild-type N2 (obtained from *Caenorhabditis* Genetics Center), *Cehcf* deletion mutant *Cehcf* (pk924) (a gift from Winship Herr, University of Lausanne, Switzerland). *sir-2.1* deletion mutant *sir-2.1* (ok434) has a truncation at position 270 in the protein sequence resulting from 768 bp deletion of part of the exon V and most of intron V, which in turn leads to frame shift and earlier stop codon [58] (obtained from *Caenorhabditis* Genetics Center); *sir-2.1* overexpression mutant *sir-2.1* (LG100) has 2.2-kilobase (kb) genomic fragment of sir-2.1 integrated into the N2 wildtype genome [28] (obtained from *Caenorhabditis* Genetics Center).

All strains were cultured using standard methods [75]. Unless otherwise stated, NGM plates were seeded with E. coli OP50 as the food source.

Lifespan assays

Before RNAi lifespan assays, RNAi plates are prepared by seeding RNAi bacteria, which were grown in Luria broth with 100 μg/ml ampicillin at 37° C for 10−16 h, onto NGM plates containing 4 mM IPTG [76]. FUDR-RNAi plates are prepared as RNAi plates with 50 μg/ml FUDR to prevent the growth of progeny. 20-40 young adults were picked and allowed to lay eggs on RNAi plates at 25℃ for 5 hours, and then parents were killed by burning. The progeny were grown on RNAi plates at 25℃ until they developed into young adult stage. The young adult worms were then transferred to FUDR-RNAi plates.

In lifespan assays using NGM plates, NGM plates were seeded with OP50 and FUDR-NGM plates are prepared as NGM plates with 50 μg/ml FUDR to prevent the growth of progeny. 20-40 young adults were picked and allowed to lay eggs on NGM plates at 25℃ for 5 hours, and then parents were killed by burning. The progeny were grown on NGM plates at 25℃ until they developed into young adult stage. The young adult worms were then transferred to FUDR-NGM plates.

For both lifespan assays, plates were incubated at 25℃. Number of death were recorded every day (worms failed to respond to a gentle prodding with a platinum wire were scored as dead). We defined the day 0 as the day when we transferred the young adult worms. Statistical analysis was done using the SPSS software and p-values were calculated using the log-rank test. All the lifespan experiments were repeated at least two independent times.

Transgenic animals

A GFP-fused *Cehcf* plasmid (Lab stock, Figure 3-1) was injected into N2 wild type worms at 90 ng/ml along with pRF4 at 10 ng/ml [77] to obtain stable extrachromosomal transgenic lines YD1 (*pCehcf*::*Cehcf*::*gfp*; *rol-6*). Lines were maintained by picking roller animals.

The GFP-fused *Cehcf* plasmid was injected into LG100 mutants at 90 ng/ml to obtain stable extrachromosomal transgenic lines YD2 (*sir-2.1 o. e.* (LG100); *pCehcf*::*Cehcf*::*gfp*; *rol-6*). Lines were maintained by picking GFP positive worms under fluorescence microscope.

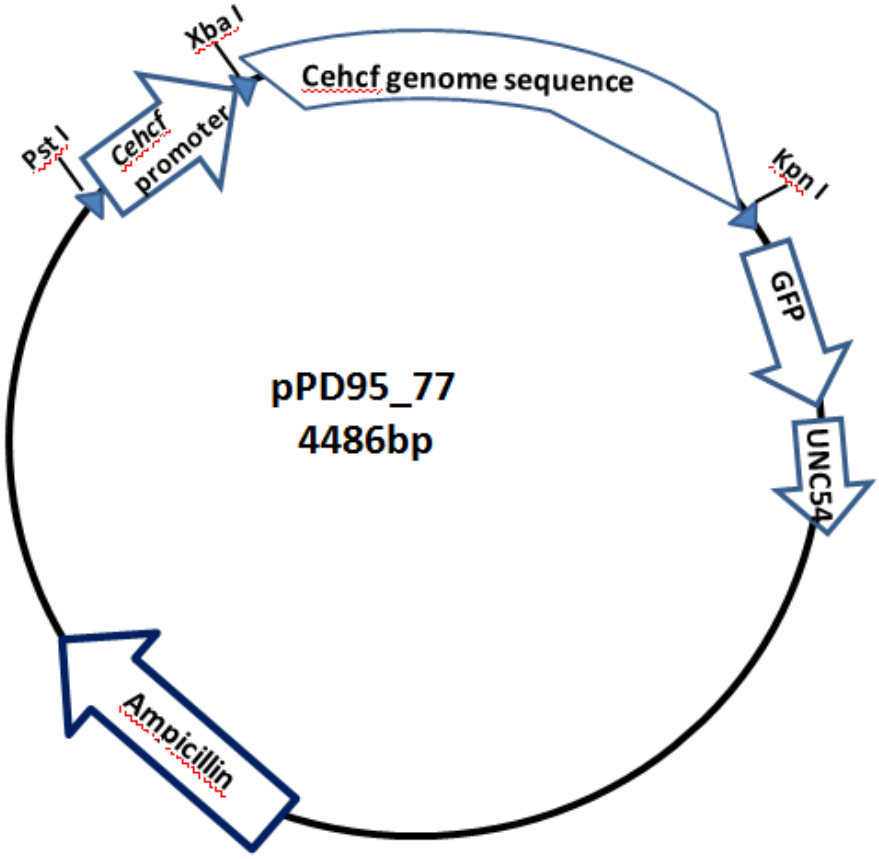


Figure 3-1. Schematic structure of plasmid CeHCF-GFP used in microinjection. A GFP-fused *Cehcf* plasmid (*PCehcf*::*Cehcf*::gfp) was created by inserting *Cehcf* promoter (～500bps) and entire genomic coding region of *Cehcf* (～3.1kb) into the pPD95_77 plasmid (～4.5kb).

RNA isolation and qRT-PCR

Eggs are collected from well maintained worms by synchronization and grown on RNAi plates for 4-5 days (about two generations) at 25°Cand then rinsed off by M9 buffer for RNAi isolation. Total RNA from 15-20 μl of worm pellet was isolated using Absolute RNA Miniprep Kit (obtained from Agilent Technologies). cDNAs were synthesized with Oligo dT using SuperScript III

First-Strand Kit (Invitrogen, USA). qRT-PCR reactions were performed using iQ SYBR Green Supermix (BIO-RAD, USA) and the iCycler iQ™ Real-Time PCR Detection System (BIO-RAD, USA). The qRT-PCR conditions were: (1) 95℃ for 3 min, (2) 40-cycles of 10 s at 95℃ and 30 s at 60℃, (4) at the end, melting curve analysis was performed for each primer set to ensure the specificity of the amplified product. For qRT-PCR, *act-1* was used as the internal control, and the RNA level of each gene of interest was normalized to the level of *act-1* for comparison. The qRT-PCR experiment was repeated at least three times using independent RNA/cDNA preparations. The data were pooled and analyzed using Student's t-test.

qRT-PCR primers

The qRT-PCR primers sequences are: for *act-1* are: (1) forward primer: 5'-CCAGGAATTGCTGATCGTATGCAGAA-3'; (2) reverse primer: 5'-TGGAGAGGGAAGCGAGGATAGA-3' (product length: 133 bp). Primers for *sod-3* are: (1) forward primer: 59-CCAACCAGCGCTGAAATTCAATGG-39; (2) reverse primer: 5'-GGAACCGAAGTCGCGCTTAATAGT-3' (product length: 127 bp).

Antibodies and Westernblot

The crude anti-CeHCF antiserum (gift from Dr. Sylvia Lee lab) was subsequently purified using S-tagged CeHCF. In brief, the crude anti-CeHCF antiserum was incubated with purified S-tagged CeHCF immobilized on nitrocellulose membrane. Poorly bound proteins were removed by multiple washes in TBS buffer, and the bound anti-CeHCF antibody was recovered by subsequent elution.

For immunoblotting, affinity purified anti-CeHCF antibody was used as the primary

antibody (1:1000) and donkey anti-guinea pig IgG (obtained from Fitzgerald Industries International, 1:10000) was used as the secondary antibody. For ACTIN immunoblotting, anti-ACTIN (mouse, obtained from DSHB) was used as the primary antibody (1:3000) and goat anti-mouse IgG (obtained from Genscript, 1:10000) was used as the secondary antibody. Westernblot is performed following standard protocol [78].

CHAPTER FOUR

RESULTS

Cehcf modulates lifespan in a *sir-2.1*-dependent manner

Cehcf was first identified as a novel aging regulator gene in the genome-wide RNAi screening [79], and its longevity function is linked to DAF-16 [31]. The mammalian homolog of CeHCF, HCF-1, was essential in stabilizing the transcriptional complex involving the herpes simplex virus (HSV) VP-16 transcription factor, as well as in regulating cell cycle progression [64-66]. CeHCF has been demonstrated to share similarity with mammalian HCF-1 both structurally and functionally [72-74]. Thus the function of CeHCF in longevity may also act, as HCF-1, by assembling different protein complexes in different gene expression regulation [73].

Considering the close cooperation between SIR-2.1 and DAF-16, the epistatic relationship between CeHCF and SIR-2.1 was tested. A *sir-2.1* null mutant *sir-2.1*(*ok434*) was obtained, which has a truncation at position 270 in the protein sequence resulting from a 768 bp deletion of part of the exon V and most of intron V, which in turn leads to a frame shift and earlier stop codon [58]. Results show that *Cehcf* RNAi could extend the lifespan of wildtype N2 worms (15.66 $\pm$0.23 days with *Cehcf* RNAi and 14.51 $\pm$ 0.2 days with control RNAi, $p < 0.05$), while the same treatment fails to extend the lifespan of deletion mutant *sir-2.1* (ok434) (14.61$\pm$0.36 days with *Cehcf* RNAi and 14.93$\pm$0.31 days with control RNAi, $p>0.05$). Thus the aging regulation function of CeHCF also requires the presence of SIR-2.1 (Figure 4-1, Table 4-1).

Table 4-1. The lifespan extension caused by *Cehcf* RNAi could be fully suppressed by loss of *sir-2.1*.

Strains (RNAi)	Mean Lifespan	Std Error	Number of worms	p-value (Compared to control RNAi)
N2 (Control RNAi)	14.51	0.2	118	
N2 (*Cehcf* RNAi)	15.66	0.23	80	0.0003
sir-2.1 (ok434) (Control RNAi)	14.95	0.31	39	
sir-2.1 (ok434) (*Cehcf* RNAi)	14.61	0.36	41	0.6387

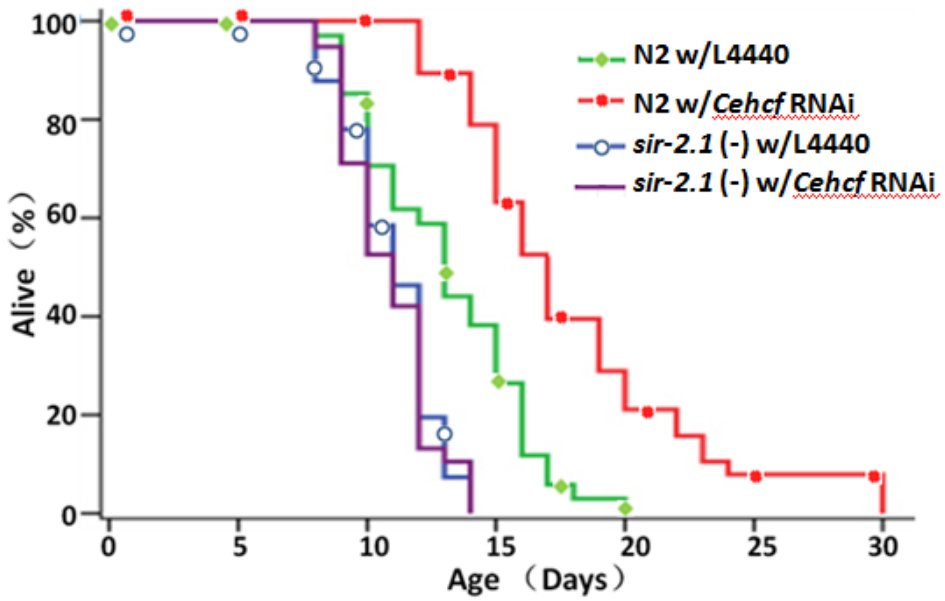


Figure 4-1. *Cehcf* affects life span in a *sir-2.1* dependent manner. Lifespan assay was performed in 25℃ and data was pooled from at least 2 independent trails. Wildtype N2 worms lives 15.66 ±0.23 days with *Cehcf* RNAi and 14.51 ± 0.2 days with control RNAi ($p < 0.05$), while the same treatment fails to extend lifespan of deletion mutant *sir-2.1* (ok434) (14.61±0.36 days with *Cehcf* RNAi and 14.93±0.31 with control RNAi, $p > 0.05$).

Loss of *Cehcf* fails to further extend longevity caused by *sir-2.1* overexpression

To further confirm the CeHCF/SIR-2.1 genetic pathway, the sir-2.1 overexpression strain *sir-2.1* (LG100) was obtained, which has a 2.2-kilobase (kb) genomic fragment of sir-2.1 integrated into the N2 wildtype genome [28]. Previous study shows that the *sir-2.1* (LG100) has a significantly longer lifespan than wildtype N2 worms [28]. If CeHCF and SIR-2.1 are in the same

genetic pathway, loss of CeHCF should fail to extend the longevity of *sir-2.1* (LG100).

Indeed, the data indicates that with extra copies of SIR-2.1, *sir-2.1* (LG100) lives longer than the N2 wildtype (13.307 $\pm$ 0.26 days for N2 wildtype fed with control RNAi, and 16.99 $\pm$ 0.331 for LG100 fed with control RNAi, $p < 0.05$), but *Cehcf* RNAi could not extend the lifespan of *sir-2.1* overexpression mutant (16.99 $\pm$ 0.331 days for LG100 fed with control RNAi, and 16.591$\pm$0.32 days for LG100 fed with *Cehcf* RNAi, $p > 0.05$). This result further confirms the epistatic relationship between CeHCF and SIR-2.1. Together with the previous result that the longevity caused by loss of CeHCF could be suppressed by loss of SIR-2.1, it indicates that CeHCF might act genetically upstream of SIR-2.1 (Figure 4-2, Table 4-2).

Table 4-2. *Cehcf* RNAi fails to further extend lifespan of long-lived *sir-2.1* overexpression mutant.

Strains (RNAi)	Mean Lifespan	Std Error	Number of worms	p-value (Compared to control RNAi)
N2 (Control RNAi)	13.307	0.260	88	
N2 (*Cehcf* RNAi)	16.170	0.333	100	< 0.0001
sir-2.1 (LG100) (Control RNAi)	16.990	0.331	100	
sir-2.1 (LG100) (*Cehcf* RNAi)	16.591	0.320	93	0.242

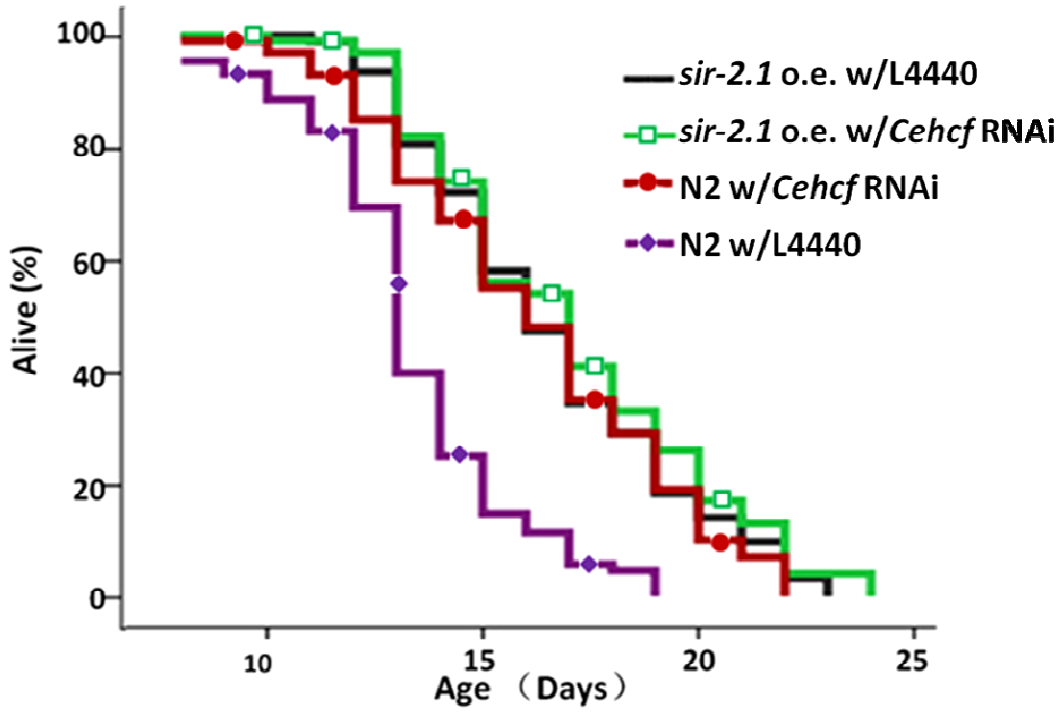


Figure 4-2. *Cehcf* RNAi fails to further extend the lifespan of long-lived SIR-2.1 overexpression mutant. SIR-2.1 overexpression mutant *sir-2.1* (LG100) lives significant longer than the N2 wildtype (13.307 N2 wildtype fed with control RNAi, and 16.99 days for LG100 fed with control RNAi, p 0.05), but *Cehcf* RNAi fails to further extend the lifespan of *sir-2.1* overexpression mutant (16.99 days for LG100 fed with control RNAi, and 16.591 0.32 days for LG100 fed with *Cehcf* RNAi, p 0.05). Overall, it suggests CeHCF might acts genetically upstream of *sir-2.1*. Lifespan assay was performed in 25 and data was pooled from at least 2 independent trails.

***Cehcf* overdosage could suppress longevity caused by *sir-2.1* overexpression**

Since loss of CeHCF could extend lifespan in *C.elegans*, it is interesting to see whether

increased level of CeHCF could decrease the lifespan. Thus a CeHCF overexpression mutant YD1 was generated (Figure 4-3) by microinjection with CeHCF::GFP plasmid (Figure 3-1) and then performed lifespan assay. Results show that with extra copies of CeHCF, YD1 has a slightly reduced lifespan comparing to the N2 wildtype (14.04 0.266 days for N2, and 13.388 0.24 days for YD1, p 0.05) (Figure 4-4, table 4-3).

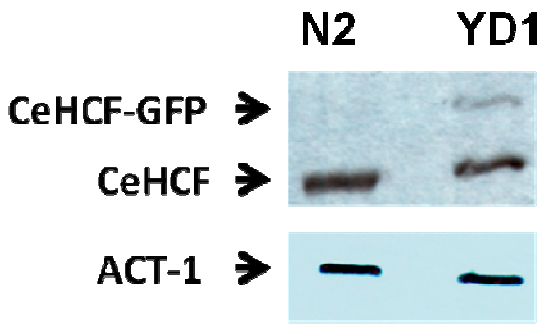
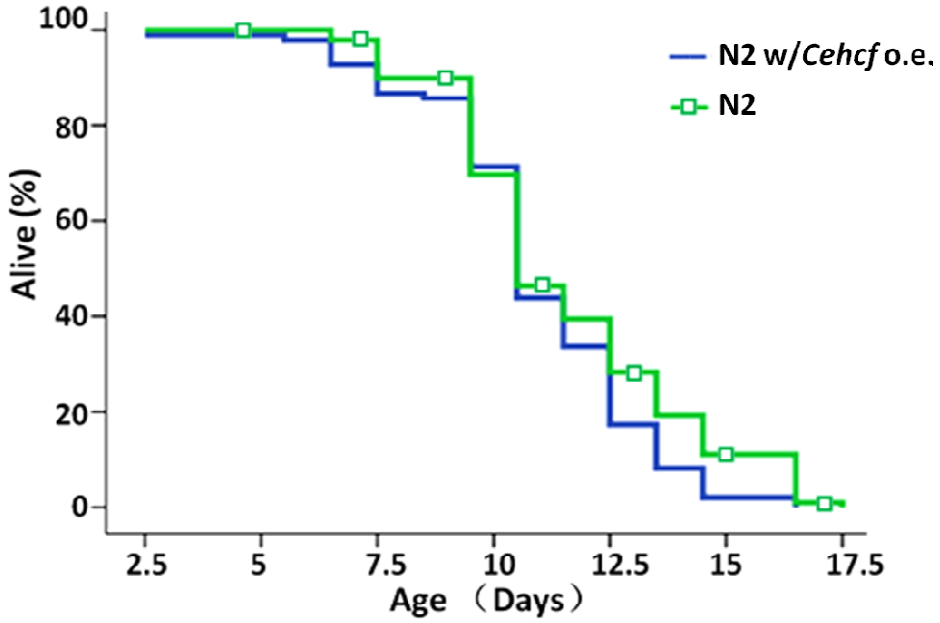


Figure 4-3. Western blot test shows GFP fusioned CeHCF is expressed in mutant strain YD1. Endogenous CeHCF is about 80 kD and GFP fusioned CeHCF is about 120kD. Purified anti-CeHCF antibody is used as primary antibody and donkey anti-guinea pig IgG was used as the secondary antibody. ACT-1 is used as the loading control.

Table 4-3. *Cehcf* overexpression could slightly shortened lifespan of wildtype worm.

Strains	Mean Lifespan	Std Error	Number of worms	p-value (Compared to N2)
N2	14.040	0.266	99	
YD1 (*Cehcf* o.e.)	13.388	0.240	98	0.049



Fugure 4-4. Overexpression of CeHCF in the wildtype worm could slightly shortened its lifespan. CeHCF overexpression mutant was generated by microinjection using the plasmid of GFP fusioned CeHCF. CeHCF overexpression mutant YD1 lives 13.388 0.24 days, and wiltype N2 worm lives 14.04 0.266 days. There is a slight lifespan reduction caused by overexpression of CeHCF comparing to the N2 wildtype (p 0.05).

Next, the possibility that CeHCF negatively regulates SIR-2.1 to mediate lifespan was tested. To investigate this assumption, LG100 is injected with the same CeHCF::GFP plasmid as in YD1 to obtain the *Cehcf/sir-2.1* co-overexpression strain YD2 (Figure 4-5). The following lifespan assay indicated that the Ce*hcf* overexpression could completely suppress the lifespan extention in SIR-2.1 overexptression mutant LG100. The average lifespan of YD2 is 11.448 0.333, while the average lifespan of *sir-2.1* (LG100) is 15.359 0.368 (p 0.05) (Figure 4-6, Table 4-4).

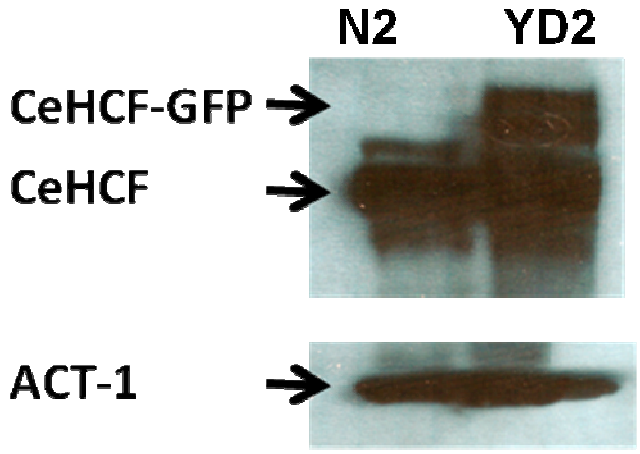


Figure 4-5. Western blot test shows GFP fusioned CeHCF is expressed in mutant strain YD2. Endogenous CeHCF is about 80 kD and GFP fusioned CeHCF is about 120kD. Purified anti-CeHCF antibody is used as primary antibody and donkey anti-guinea pig IgG was used as the secondary antibody. ACT-1 is used as the loading control.

Table 4-4. The lifespan extension caused by SIR-2.1 overexpression could be fully suppressed by CeHCF overexpression.

Strains	Mean Lifespan	Std Error	Number of worms	p-value (Compared to N2)	p-value (Compared to LG100)
N2	12.940	0.280	117		<0.0001
LG100 (*sir-2.1* o.e.)	15.359	0.368	131	<0.0001	
YD2 (*sir-2.1 o.e./Cehcf* o.e.)	11.448	0.333	105	0.016	<0.0001

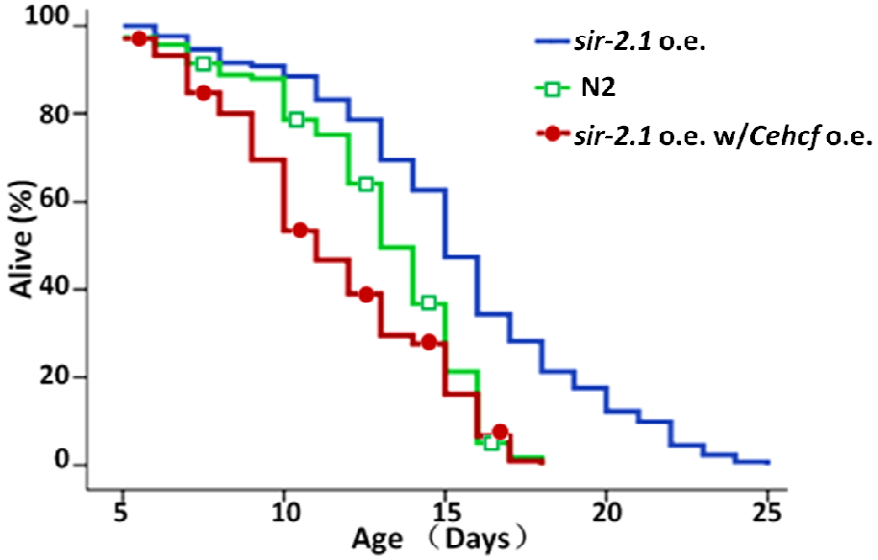


Figure 4-6. Overexpression of CeHCF fully suppressed the lifespan extension caused by SIR-2.1 overexpression. The average lifespan of CeHCF/SIR-2.1 double overexpression mutant YD2 is 11.448 0.333 days, while the average lifespan of SIR-2.1 overexpression mutant *sir-2.1* (LG100) is 15.359 0.368 days. CeHCF/SIR-2.1 double overexpression mutant YD2 lives significantly shorter than the SIR-2.1 overexpression mutant *sir-2.1* (LG100) (p 0.05). Lifespan assay was performed in 25 and data was pooled from at least 2 independent trails.

Cehcf acts as a gene-specific co-regulator of DAF-16

Previous studies show that CeHCF mediates transcriptional activity of DAF-16 [31], and meanwhile extra copies of *sir-2.1* could increase the stress resistance in DAF-16 dependent manner. Therefore, it is interesting to explore if CeHCF cooperates with SIR-2.1 to regulate the DAF-16 target gene. *sod-3*, one major mitochondrial superoxide dismutases (SODs) in *C.elegans*, was choose as the target gene, which indicates the transcriptional activity level of DAF-16. *act-1*

was chosen as the internal control. Previous research showed that disruption of the major mitochondrial sod-genes decreased survival in the presence of paraquat, juglone or hyperbaric oxygen [80], although it did not affect the lifespan of *C.elegans* in unstressed condition [80]. *sod-3* is a direct target of DAF-16, which requires the beta-catenin BAR-1 to promote sod-3 expression and oxidative stress resistance [20]. In *Cehcf* deletion mutants, *sod-3* has a significantly higher transcriptional level than in wildtype [31]. Recent studies also showed that *sir-2.1* overexpression could increase *sod-3* level by using an exogenous GFP reporter [27]. Together with the results that the lifespan extension resulting from loss of CeHCF could be abolished by loss of SIR-2.1, it is plausible to assume depletion of SIR-2.1 might also suppress the activation of DAF-16 by loss of CeHCF. Additionally, our results show that *Cehcf* RNAi could not further extend the lifespan of *sir-2.1* overexpression strain LG100, so it would be interesting to see if it also fails to increase the *sod-3* level in LG100.

N2 wildtype, deletion mutant *sir-2.1* (ok434) and overexpression mutant *sir-2.1* (LG100) were fed on *Cehcf* RNAi and control RNAi. Mix stage worms were collected for total mRNA isolation and reverse transcription. qRT-PCR revealed that *Cehcf* RNAi treatment increases the *sod-3* transcriptional level by 2.5$\pm$0.006 fold compared to control RNAi ($p<0.05$) in N2 wildtype, and in the deletion mutant *Cehcf* (*pk924*), *sod-3* transcriptional level is elevated 3.8$\pm$0.009 fold compared to wildtype ($p< 0.05$). However, *Cehcf* RNAi slightly increased the *sod-3* level in *sir-2.1*(ok434) deletion mutant and *sir-2.1* (LG100) overexpression strain. Compared to N2 wildtype, loss of SIR-2.1 alone caused a 2.3$\pm$0.0014 fold increase of sod-3 level ($p=0.0555$), and loss of both SIR-2.1 and CeHCF resulted in a 4.4$\pm$0.019 fold increase of *sod-3* ($p= 0.0539$) (Figure

4-7, table 4-5). With extra copies of SIR-2.1, *sir-2.1* (LG100) exhibited a 2.0±0.011 fold change of *sod-3* level compared to N2 wild type, although this change was not statistically significant (p=0.0905) (Figure 4-7, table 4-6). Loss of CeHCF in LG100 results in a 2.2±0.005 fold change of *sod-3* level compared to N2 wildtype (p=0.0536), while it caused a 1.1±0.01 fold change compared to LG100 fed with control RNAi (p=0.1858).

4-5. *sod-3* transcriptional level could be increased by loss of *sir-2.1* and *Cehcf*

Strain (RNAi)	Fold Change with SEM (Compared to N2*[a])	Fold Change (Compared to Control RNAi*[b])	p-value (Compared to N2*[a])	p-value (Compared to Control RNAi*[b])
N2 Wildtype (Control RNAi)				
N2 Wildtype (*Cehcf* RNAi)	2.5±0.006	2.5±0.006		0.0149
sir-2.1 (-) (ok434) (Control RNAi)	2.3±0.0014		0.0555	
sir-2.1 (-) (ok434) (*Cehcf* RNAi)	4.4±0.019	1.9±0.019	0.0539	0.1779
Cehcf (-) (PK924) (Control RNAi)	3.8±0.009		0.008	

*a. Comparison is between mutants and N2 wildtype.

*b. Comparison is between different RNAi treatments in the same strain.

Table 4-6. *sod-3* transcriptional level could be increased by *sir-2.1* overexpression

Strain (RNAi)	Fold Change with SEM (Compared to N2*[a])	Fold Change (Compared to Control RNAi*[b])	p-value (Compared to N2*[a])	p-value (Compared to Control RNAi*[b])
N2 (Control RNAi)				
N2 (*Cehcf* RNAi)	2.5±0.006	2.5±0.006		0.0149
sir-2.1(LG100) (Control RNAi)	2.0±0.011		0.0905	
sir-2.1(LG100) (*Cehcf* RNAi)	2.2±0.005	1.1±0.01	0.0536	0.1858
Cehcf (PK924) (Control RNAi)	3.8±0.009		0.008	

*a. Comparison is between mutants and N2 wildtype.

*b. Comparison is between different RNAi treatments in the same strain.

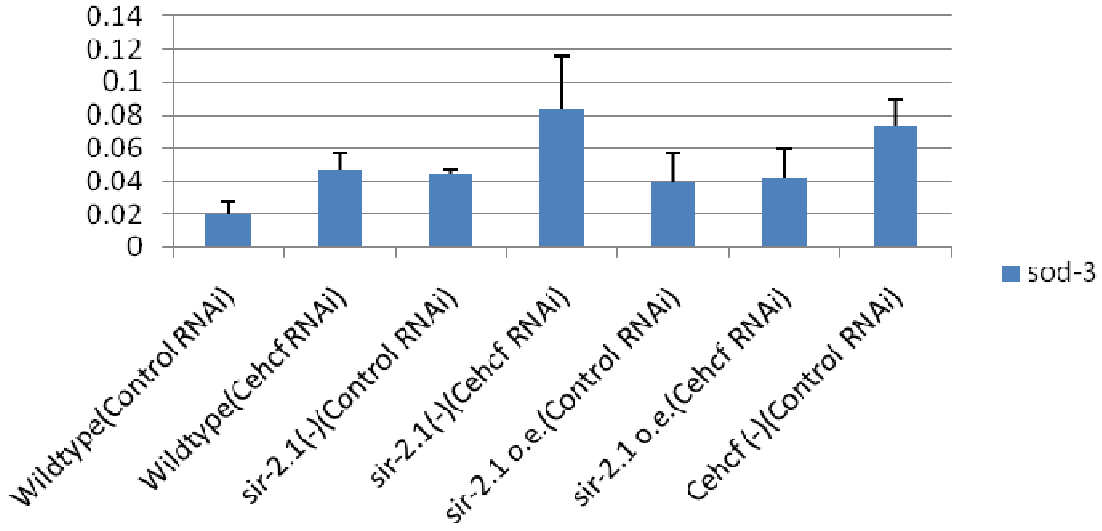


Figure 4-7. *sod-3* is not the direct target co-regulated by CeHCF and SIR-2.1. Average *sod-3* transcriptional level is normalized to level of *act-1. Cehcf* RNAi increases the *sod-3* transcriptional level by 2.5 0.006 fold comparing to control RNAi (p 0.05) in N2 wildtype, and in deletion muant *Cehcf* (*pk924*), *sod-3* transcriptional level is elevated 3.8 0.009 folds comparing to wildtype (p 0.05). This result is consistent with previous research [21]. However, both *sir-2.1* deletion mutant and overexpression mutant shows an increased level of sod-3 (2.3 0.0014 fold increase in deletion mutant *sir-2.1* (ok434), and 2.0 0.011 fold increase in overexpression mutant *sir-2.1* (LG100) comparing to N2 wild type), which suggests that the change of transcriptional level of sod-3 may be caused by indirect effects of SIR-2.1.

The higher level of *sod-3* in both *sir-2.1* deletion mutant *ok434* and *sir-2.1* overexpression mutant LG100 indicates that *sod-3* may not be the specific target regulated by the SIR-2.1/DAF-16 pathway. The variation of the *sod-3* level possibly resulted from the indirect

influences by the change of CeHCF and SIR-2.1. Previous research in yeast cells also reported that *sir2* deletion mutants showed increased expression of many genes involved in DNA repair and stress resistance [57]. Therefore, *sir-2.1* and *Cehcf* might be involved in limiting the DNA binding specificity of DAF-16 to a quite narrow range: loss of either of them may alter the specificity of DAF-16, which indirectly affect DAF-16 downstream targets.

CHAPTER FIVE

DISCUSSION

In this study, we have identified *Cehcf/sir-2.1/daf-16* as a novel genetic pathway in aging regulation (Figure 5-1). Loss of CeHCF by RNAi or in genetic mutants could extend lifespan in DAF-16 dependent [31] and SIR-2.1 dependent manner. *Cehcf* RNAi fails to further extend lifespan of the long-lived *sir-2.1* overexpression mutant LG100, which further confirms the epistatic relationship between *Cehcf* and *sir-2.1*. Also, extra copies of CeHCF could fully suppress the long lived phenotype of the *sir-2.1* overexpression mutant, which suggests *Cehcf* may negatively regulate SIR-2.1. Overall, our results suggest that CeHCF acts genetically upstream of SIR-2.1 and affects function of DAF-16 by negatively regulating SIR-2.1. Our results also give some insights to DAF-16 mediated transcription. Increased resistance to oxidative stress and heavy mental stress are reported with a lack of CeHCF and can be abolished by depletion of DAF-16 [31]. SIR-2.1 overexpression is also reported to promote *daf-16*-dependent transcription by using an exogenous GFP-reporter [27]. However, *sod-3* may not be co-regulated by CeHCF and SIR-2.1. The level of change of *sod-3* may just be a reflection of the alteration of DAF-16 DNA binding specificity, which might be narrowed by CeHCF and SIR-2.1 in nature condition.

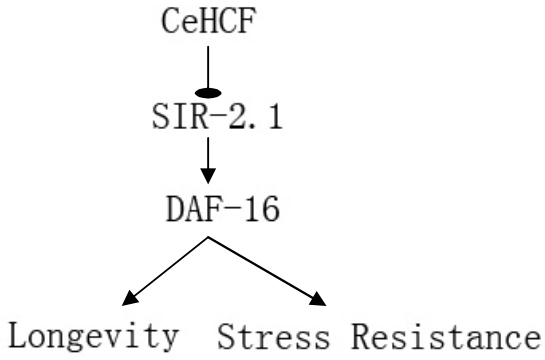


Figure 5-1. CeHCF negatively regulates SIR-2.1 to mediate target specificity of DAF-16 and Lifespan. Lifespan extention caused by depletion of CeHCF could be fully suppressed by losing either DAF-16 or SIR-2.1. SIR-2.1 overexpression mutant with CeHCF depletion displays similar lifespan as without CeHCF depletion, which further suggests CeHCF acts genetically upstream of SIR-2.1. Lifespan elongation caused by SIR-2.1 overexpression could be fully suppressed by overexpression of CeHCF, which supports that CeHCF negatively regulates SIR-2.1.

Recent research show that DAF-16 could be able to regulate various subsets of downstream genes, only a small part of which would be affected by CeHCF [31]. These findings indicate that CeHCF acts as a gene-specific co-factor of DAF-16. The putative co-regulator function of *C. elegans* host cell factor is consistent with the known role of mammalian HCF-1 in gene expression regulation. Human HCF-1 was originally identified to be an essential host cell factor required for the assembly of the transcriptional complex involving the herpes simplex virus

VP16 transcriptional activator [64]. In the context of normal cellular function, mammalian HCF-1 has been shown to interact with many transcription and chromatin factors {65, 67, 68] via its versatile domains [70, 71]. Using an immunoprecipitation/mass spectrometry strategy, human HCF-1 has been shown to associate with two histone modifying complexes with opposing effects on gene expression. HCF-1, via the Kelch domain, associates with the trithorax-related Set1/Ash2 lysine 4 histone 3 methyltransferase (HMT), a complex often involved in gene activation, and via the basic region, associates with the Sin3 histone deacetylase complex (HDAC), a complex often involved in gene repression [67]. Moreover, human HCF-1 is found to associate with the transcriptional activator E2F1 and the transcriptional repressor E2F4, two major regulators that act at slightly different times during the G1/S phase of the cell cycle [68]. Interestingly, HCF-1 is shown to recruit the Set1/Ash2 HMT activating complex to E2F1 and the Sin3 HDAC repressing complex to E2F4 at the appropriate times of the cell cycle, which likely helps to reinforce the activating or repressing functions of the respective E2F family members [68]. These results together indicate that mammalian HCF-1 likely functions as a scaffolding protein that brings together appropriate chromatin factors and DNA binding transcription factors to regulate gene expression.

Interestingly, our results show that CeHCF forms a complex with DAF-16 [31] and SIR-2.1 [our lab data] in *C.elegans*. We also identified that the conserved Kelch domain may also be important for the association between CeHCF and DAF-16, or CeHCF and SIR-2.1 by the in *vitro* pull down experiment (our lab data). CeHCF is co-localized with DAF-16 and SIR-2.1 in the nervous system and hypodermis [31, 58]. However, neither the DAF-16/SIR-2.1 protein complex,

nor the three protein complex (our lab data) could be detected in unstressed condition [27]. So it is likely that the CeHCF/DAF-16 complex and the CeHCF/SIR-2.1 complex are individually existing in the nucleus in unstressed condition. However, in *C. elegans*, SIR-2.1 is thought to bind to DAF-16 and promote DAF-16-regulated transcription upon heat shock. Therefore, our results are able to support this possibility that CeHCF acts by somehow repressing the regulatory activity of SIR-2.1, in turn regulating the transcriptional specificity of DAF-16. In the future, it would be interesting to investigate whether CeHCF regulates DAF-16/SIR-2.1-mediated transcription by recruiting additional chromatin factors, such as the Sin3 HDAC complex in *C. elegans*.

The involvement of SIR-2.1, a well-established DAF-16 cofactor, in the longevity mechanism of CeHCF, brought more insights as well as questions. SIRT1, the mammalian homolog of SIR-2.1, is able to deacetylate FOXO3a, FOXO4 and FOXO1 in mammalian cells, but this interaction between SIRT1 and FOXO3a could repress the P300-mediated activation of FOXOs, resulting in the lower level of FOXO target gene transcription [29, 30]. These targets are largely involved in proapoptosis [29, 30]. It is possible that slowed apoptosis caused by SIRT1 might benefit the long-term survival by alleviating the progressive erosion of organ systems with age. Studies on the crystal structure of FOXO1 have also revealed that DNA binding affinity of FOXO1 could be reduced by acetylation of Lys245 and Lys248 by CBP/p300 [52, 53]. Therefore, it is possible that removal of the acetyl group by deacetylase could restore high DNA binding affinity on certain sites, which seems to conflict with the observation mentioned above. One possible explanation might be that after acetylation or deacetylation, there are further modifications on FOXOs, and/or interactions with various cofactors, which affects specificity of FOXOs and leads to

changed transcriptional level of corresponding genes. Our results could support this hypothesis that CeHCF may affect lifespan by negatively regulating SIR-2.1 resulting in an altered specificity of DAF-16. Thus, it is possible that the deacetylase enzyme activity of SIR-2.1 is important for the longevity function of CeHCF. It will be interesting to see if SIR-2.1 with an inactivated deacetylase function could affect CeHCF mediated lifespan regulation.

Another interesting question about SIR-2.1 is derived from studies of yeast homolog SIR2: researches show that SIR2 may play different roles in regulating yeast replicative lifespan and chronological lifespan, which may represent models for mitotic cells and post-mitotic cells respectively [24, 25, 57]. In multi-cellular organisms, such as *C. elegans*, number and location of mitotic cells and post-mitotic cells change along with the development process. Meanwhile, the modification pattern of CeHCF alters along with the development [72]. Thus it is plausible to assume that CeHCF may have different functions in different developmental stages. Indeed, previous data shows that in cold-temperature loss of CeHCF protein would cause embryonic lethality and early embryonic mitotic, which indicates its function in development [71, 72]. Our lab data could also indirectly support this hypothesis: a big RNA level variation occurred on certain DAF-16 target genes when we sampled *C.elegans* of mixed stages (our lab data). Interestingly, all those genes could affect developmental process, resulting in developmental timing variation, slow growth, larval lethal and sterile progeny; therefore, the variation could possibly be due to the different proportion of each stage in the sample. Future studies on how the CeHCF/SIR-2.1 regulates DAF-16-mediated transcription in each specific developmental stage will reveal more details for the mechanism behind CeHCF longevity function.

The CeHCF is also involved in stress response mediated by DAF-16: loss of CeHCF increased resistance of paraquat and cadmium exposure [31]. Therefore, it is possible that under stress conditions, living cells might try to lower the level of CeHCF in order to facilitate survival. However, after testing the CeHCF level under different stress condition, we found out it is not the case. The CeHCF level remains constant in response to different intensities of heat shock stress and oxidative stress (our lab data). This result further leads us to an alternative model that the presence of protein complexes DAF-16/CeHCF and SIR-2.1/CeHCF may undergo a dynamic change in response to stress. Indeed, the post-modification of DAF-16 largely depends on its upstream stimulus. It is plausible to assume that germline signal, the only proliferative tissue, which also goes through DAF-16 [27], might link to the longevity function of CeHCF, because *Cehcf* deficiency could cause low brood size and slow growth (data not shown), which indicates the role of *Cehcf* in regulation of cell proliferation. However, loss of Cehcf continues to extend the lifespan of germline deficiency mutants, which rules out the epistatic relationship between CeHCF and germline signaling [31]. The lifespan extension caused by extra copies of sir-2.1 requires 14-3-3 proteins and DAF-16 [27, 28], which means the nucleus 14-3-3 proteins may help forming DAF-16/SIR-2.1 complex. However, it was also reported that 14-3-3 proteins could regulate lifespan independent of DAF-16 [81], which makes the longevity mechanism behind 14-3-3 more complicated. Since overexpression of CeHCF could suppress the longevity caused by extra copies of *sir-2.1*, it is necessary to investigate whether 14-3-3 is also involved in the CeHCF/SIR-2.1/DAF-16 pathway. We tested the lifespan of 14-3-3 deletion mutants fed with *Cehcf* RNAi. Results showed that Cehcf RNAi was still able to extend lifespan even in the absence of

14-3-3 proteins, which suggests they may be in different pathways (our lab data).

Depletion of *Cehcf* only decreases the sensitivity to oxidative stress and heavy mental stress, which again confirms the idea that CeHCF may limit the specificity of DAF-16 [31]. Post-modifications of DAF-16 caused by upstream stimulus of those stress may affect the interaction of CeHCF/DAF-16 and/or CeHCF/SIR-2.1 complex. Upon oxidative stress, CST-1 kinase, a homolog of mammalian MST1 in *C. elegans*, promotes nuclear translocation of DAF-16 by phosphorylation [15]. This distinct modification recruits another co-activator of DAF-16, beta-catenin *bar-1*, resulting in increased transcription of *sod-3* [20]. Since CeHCF is also specifically involved in DAF-16 mediated oxidative stress response, it could be interesting to see if the longevity function of CeHCF is also related to CST-1 and BAR-1. Indeed, structure analysis of FOXO1 shows that MST1 phosphorylation would surprisingly lower the DNA binding affinity of FOXO1, which opposes the previously reported longevity and stress resistance promoting function of MST1 [15, 52]. Thus, it is necessary to have further modifications on the nuclear DAF-16 in order to restore its DNA binding ability while CeHCF, the only reported negative regulator of DAF-16, might interfere with this process. Our data also suggests that CeHCF/SIR-2.1 could affect DAF-16 mediated transcription, but their direct targets are not clear. Candidates co-regulated by CeHCF/SIR-2.1 are highly likely to be involved in paraquat induced oxidative stress response. Further clarification of those direct targets would greatly help to reveal mechanism behind CeHCF/SIR-2.1/DAF-16 pathway.

Previous studies about the tissue specific location of DAF-16, SIR-2.1 and CeHCF suggest that the CeHCF/DAF-16 complex may be present in the intestinal cells without SIR-2.1 while the

CeHCF/SIR-2.1 complex may be in the pharyngeal alone [31, 58]. Thus it is still unclear that whether CeHCF/SIR-2.1 complex is involved in other regulation mechanisms as well. Indeed, SIR-2.1 is also hypothesized to function as metabolism switch, which is activated by a high NAD/NADH ratio [82]. The latest research shows that KAT-1, an enzyme functioning in fatty acid oxidation, is required for the extension of lifespan and enhanced stress resistance in the SIR-2.1 overexpression mutant [82]. Interestingly, FAT-5, the delta-9 fatty acid desaturase, is activated by reduced *daf-2* signaling but could be repressed by loss of *Cehcf* in a *daf-16*-independent manner [31]. Thus, it is possible that CeHCF might affect fatty acid metabolism through SIR-2.1. Moreover, studies in mammalian cell lines show that DNA damage caused by etoposide could induce increased expression of SIRT1 and this increase is largely dependent on E2F1 transcriptional factor [83]. In addition, SIRT1 could deacetylate and inhibit the transcriptional activity of E2F1 with pRb, which forms a negative feedback loop with E2F1 [83]. Meanwhile, HCF-1 is able to activate E2F1 by recruiting the Set1/Ash2 histone methyltransferase complex, which might affect SIRT1 and SIRT1-mediated stress response consequently [68]. However, the *C. elegans* E2F1 homolog, EFL-1, does not conserve the HCF binding motif as its mammalian and fly homolog [68] and depletion of CeHCF fails to show a cell-cycle arrest, thus it is still unknown whether the interaction between HCF-1 and E2F1 is conserved in worms [68]. On the other hand, CeHCF is important for multivulval phenotype caused by loss of one class of gene called SynMuv B [84]. Since both lin-35 (pRb homolog) and efl-1 (E2F1homolog) are included in this class, there might be conserved relationships existing among CeHCF, pRb and E2F1 homologs in *C. elegans*.

Overall, our data revealed a novel genetic pathway that controls the aging process, where

CeHCF affects lifespan through negatively regulating SIR-2.1 and DAF-16. By physical association with DAF-16 and SIR-2.1, CeHCF acts as a gene-specific co-regulator of DAF-16. It might regulate the DNA binding specificity of DAF-16 with SIR-2.1 and consequently mediates the transcriptional level of DAF-16 targets. The CeHCF/SIR-2.1 complex is specifically involved in DAF-16 mediated oxidative stress resistance although the detailed mechanism is still unclear. Future studies focusing on how CeHCF, SIR-2.1 and DAF-16 interplay with each other under stress condition would be necessary. Further revealing the molecular basis behind CeHCF/SIR-2.1/DAF-16 pathway could greatly facilitate our understanding about aging regulation.

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